PATENT SPECIFICATION (11) 1 296 839

1 296 839

NO DRAWINGS

(21) Application No. 27352/69 (22) Filed 29 May, 1969

(21) Application No. 36528/69 (22) Filed 21 July 1969

(23) Complete Specification filed 19 May 1970

(45) Complete Specification published 22 Nov. 1972

(51) International Classification C12D 13/10 // C07G 7/028

(52) Index at acceptance C3H 3

(72) The Inventors of this invention in the sense of being the actual Devisers thereof within the meaning of Section 16 of The Patents Act 1949 are HELLE OUTTRUP, nee NIELSEN of 57 Bavnestedet, DK-3500 Værløse, OTTO ANDRESEN of 67, Studiestraede DK-1554 Copenhagen V and KNUD AUNSTRUP of 82, Skovbakken, DK-3520 Farum, Denmark, all Danish subjects.

(54) IMPROVEMENTS IN OR RELATING TO THE PREPARATION OF MICROBIAL ENZYMES

(71) We, NOVO TERAPEUTISK LABORATORIUM A/S, a Danish company of 115, Fuglebakkevej, 2200 Copenhagen N, Denmark, do hereby declare the invention, for which we pray that a patent may be granted to us, and the methods by which it is to be performed, to be particularly described in and by the following statement: —

The present invention relates to a process for the preparation of an enzyme product containing an alpha-amylase. The invention also relates to certain important uses of the products prepared by the said process and to the products themselves.

Alpha-amylases are widely used for industrial purposes where a partial hydrolysis of starch is desired. The effect may be a reduction of the viscosity of a starch gel or dissolution of starch adhering to fabric or utensils.

Specific industrial uses of alpha-amylases are desizing in the textile industry, liquefaction of brewing adjuncts or barley in the brewing industry, and liquefaction of starch in the production of dextrose or glucose syrups.

Amylases may also be incorporated in cleaning agents, e.g. detergents or dishwashing agents, with the purpose of facilitating the removal of starch-containing stains or starch-containing material adhering to utensils.

Sources of such amylase preparations may be animal, plant or microbial, a presently preferred source being *Bacillus subtilis*. The reasons for this are the good heat stability shown by the enzyme produced by this bacterium and the fairly broad pH range of stability and activity shown by the enzyme.

A disadvantage of this enzyme (and with all other known alpha-amylases) is, however, that the presence of Ca^{++} ions is necessary for the stability of the enzyme, a face which makes the use of the enzymes in the presence of Ca^{++}-sequestering agents difficult or impossible.

Furthermore, although the heat stability of the *B. subtilis* enzyme is good, a better heat stability would be extremely valuable to industry because it would allow a higher reaction temperature and thus a higher reaction rate.

Finally, *B. subtilis* amylase is unsuitable for use in cleaning agents where the pH value usually is above 9, as its activity and stability is very low at such high pH values.

It has now surprisingly been found that *B. licheniformis* will produce an amylase which is at least generally superior compared to the *B. subtilis* amylase with respect to heat stability, stability and activity at high pH values and which, contrary to what is known for all other types of alpha-amylases, is at least relatively stable in the presence of high concentrations of Ca^{++}-sequestering agents, *e.g.* tripolyphosphates or ethylenediamine tetraacetic acid (EDTA).

Thus, a large proportion of those strains of *B. licheniformis* which are available from recognized culture collections have been tested for amylase production and have been found, without exception, to produce an amylase as described above. The broadest aspect of the present invention is therefore based upon the concept that any strain of *B. licheniformis* is able to produce the amylase referred to.

In accordance with the above the invention provides a process for the production of an enzyme product containing an alpha-amylase having good activity and stability at high pH values, at low concentrations of Ca^{++} and at high temperature, which process comprises cultivating a microorganism of the species

Bacillus licheniformis in a nutrient medium for the microorganisms, whereafter, if desired, an enzyme product is isolated from the fermentation broth.

A specific embodiment of the invention relates to the use of the strain of *Bacillus licheniformis* NCIB 8061 in the present process.

Another specific embodiment of the invention relates to the use of a strain of *Bacillus licheniformis* selected from NCIB 8059, ATCC 6634, ATCC 6598, ATCC 11945, ATCC 8480 and ATCC 9945a in the above process.

The following results were obtained in comparative experiments conducted with enzymes from a representative strain of *B. licheniformis* and a commercial enzyme preparation prepared from a strain of *B. subtilis*.

	Alpha-Amylase from:	
Molecular weight:	*B. licheniformis* 10—20,000	*B. subtilis* 96,000
Temp. of max. activity at a reaction time of 10 mins. and at the pH values:		
6.2	90°C	70°C
8.8	90°C	70°C
Activity at pH 8.8 in % of activity at pH 6.2 at optimum temperature	100% (90°C)	20% (70°C)
and at 37°C	53%	9%
Residual activity after treatment in a 4% w/v solution of sodium tripolyphosphate at 50°C and pH 9 for 30 mins.	above 50%	appr. 10%
Residual activity after incubating a solution of the enzyme in de-ionized water for 120 mins. at		
75°C and pH 8.8	100%	27%
and at pH 5.9	86%	0%

Thus, it is characteristic of the new alpha-amylase enzyme prepared according to the invention that it shows good activity at high pH values, *e.g.* 9, and a good stability in the presence of sodium tripolyphosphate.

For the purpose of alpha-amylase production a strain of *Bacillus licheniformis* may be cultivated in a nutrient medium containing assimilable carbon and nitrogen together with other essential nutrients, the medium being composed in accordance with the principles of the known art. Suitable carbon sources are carbohydrates such as saccharose, glucose and starch or carbohydrate-containing materials such as cereal grains, malt, rice and sorghum. The carbohydrate concentration incorporated in the medium may vary widely, e.g. up to 25% w/v and down to 5% or even 1% w/v, but usually 10—20% w/v will be suitable; the percentages being calculated as dextrose. The preferred carbohydrate is starch or partially hydrolyzed starch.

The nitrogen source in the nutrient medium may be of inorganic and/or organic nature. Suitable inorganic nitrogen sources are nitrates and ammonium salts. Among the organic nitrogen sources quite a number are regularly used in fermentation processes involving the cultivation of bacteria. Illustrative examples are soybean meal, cotton seed meal, peanut meal, casein, corn steep liquor, yeast extract, urea and albumine. In addition, the nutrient medium should also contain the usual trace substances.

The cultivation may be carried out at a temperature between 25 and 50°C, preferably between 30 and 40°C.

As the cultivation is preferably carried out

under aerobic conditions it is usually necessary to make use of artificial aeration when growing the bacteria in fermentation tanks. The aeration rate is not critical, but usually one volume of air per volume of broth per minute is used.

In general, maximum yields of the alpha-amylase will be obtained after a cultivation time of 1 to 7 days.

Alpha-amylase-containing preparations may be prepared from the fermentation broth by methods well known in the art, *e.g.* by the manufacture of a liquid preparation by purification of the broth by centrifugation or filtration and addition of preservatives to the purified liquid; or by the manufacture of a solid preparation by addition to the purified broth of enzyme-precipitating agents such as ammonium sulfate, sodium sulfate or water-miscible organic solvents, such as ethanol or acetone, and recovering and drying the precipitate.

Amylase preparations prepared according to the invention may be used for all applications where amylase from *B. subtilis* normally is used.

Its use is particulary advantageous under one or more of the following conditions, high temperature, high pH (up to 10), low concentration of Ca^{++} (i.e. in the presence of sequestering agents such as sodium tripolyphosphate or ethylenediaminetetraacetic acid).

Examples of specific applications for the new amylase are: desizing, liquefaction of starch for preparation of starch hydrolysates and incorporation in cleaning agents for washing or dishwashing purposes.

The process of the invention is illustrated further in the following specific Examples in which the activities stated have been determined as follows:

The alpha-amylase activity against soluble starch was determined by the use of a modification of the SKB method performed at 37°C. The SKB method is described in Cereal Chemistry, *16*, 712 (1939). In our modified method we used no addition of beta-amylase and in details our method was as follows:

All reagent solutions must be prepared with distilled water.

A stock iodine solution is prepared by dissolving 11 g of iodine crystals and 22 g of potassium iodide in water and diluting to a volume of 500 ml. A dilute iodine solution is prepared by mixing 2 ml of stock iodine solution with 20 g of potassium iodide and diluting with water to a volume of 500 ml. The buffer-salt solution used has the following compositions:

9.36 g NaCl, 69.00 g KH_2PO_4, 4.80 g $Na_2HPO_4.2H_2O$ are dissolved in water and made up to 1 litre.

A buffered starch solution is prepared as follows:

6.95 g soluble starch (*e.g.* Merck, Amylum solubile, S luble Starch, Erg.B.6.) calculated as dry matter are made into a slurry with about 50 ml of water. The slurry is slowly added to about 200 ml of boiling water. After the starch is completely dissolved, the solution is cooled, transferred to a 1 litre volumetric flask, 35 ml of buffer-salt solution added and made up to the mark with water. Finally the solution is saturated with toluene. The pH of the finished solution is adjusted to 6. The starch solution must be as freshly prepared as possible, but can be stored in the refrigerator for not more than 24 hours.

20 ml of the buffered starch solution are measured in a test tube (diameter 24 mm, length 190 mm) and placed in a water thermostat at a temperature of 37°C. After a few minutes pre-warming add 10 ml of the amylase solution to be tested (or v ml amylase solution + (10 − v) ml water). The contents of the tube are thoroughly mixed and at the same time a stop-watch is started. At appropriate time intervals 1 ml of the reaction mixture is added to 5 ml of the dilute iodine solution, shaken and transferred to a comparison tube, and the colour is compared with the standard colour. If the colour end-point is reached in less than 10 minutes, a more dilute amylase solution or a smaller volume of amylase solution is used.

As colorimeter the Hellige Comparator 607 is used with the glass alpha-amylase standard. (cf. Redfern, Methods for determination of alpha-amylase, Cereal Chemistry *24*, 259, (1947)).

CALCULATION

Calculate the alpha-amylase activity of the sample by using the following formula:

$$A = \frac{1430 \times V}{t \times a \times v} \text{ where}$$

A = alpha-amylase activity in units per gramme or ml

t = time in minutes to reach the colour end-point

a = amount of sample expressed in grammes or ml

V = volume to which the sample is diluted (ml)

v = volume of amylase solution used (ml)

The factor "1430" is not strictly constant but depends to some degree upon the starch quality used. For exact determinations the value of the factor should be calculated by means of a standard amylase preparation with known activity.

For analysis at other pH values than the one

mentioned here a buffer system particularly suited for that pH value is used.

In each example the alpha-amylase activity at pH values from 5 to 10 is determined using the method referred to above. The value at the lower limit, *i.e.* pH 5, and the value at the upper limit, *i.e.* pH 10, is highly inaccurate, particularly the value at pH 5, where the activity curve is very steep so that a small pH variation will result in a large change in activity.

Example 1

Bacillus licheniformis NCIB 8061 was cultivated at 30°C on a rotary shaking table (220 rpm) in 500 ml baffled Erlenmeyer flasks containing 100 ml medium of the following composition:

Potato starch	40 g/l
Soybean flour	30 g/l
Ground barley	100 g/l
$CaCO_3$	5 g/l
Soybean oil	0.5 ml/l

The medium is liquefied with alpha-amylase and sterilised at 120°C for 45 minutes.

After 7 days of cultivating the amylase yield was 300 units per ml. The liquid was purified by centrifugation, 0.5% w/v $CaCl_2$ was added and pH adjusted to 7.6. The enzyme was precipitated at 2°C, by adding acetone to a concentration of 64 per cent, and then separated from the liquid by centrifugation. After drying in a dessicator, a powder with an alpha-amylase activity of 55,000 units per g was obtained.

The alpha-amylase activity against soluble starch (Merck amylum solubile) was determined at different pH values using the method described above with the use of appropriate buffer systems.

The results are expressed as per cent of the activity at pH 5.

pH	5	6	7	8	9	10
% activity	100	92	87	77	50	6

The stability of the enzyme in a solution containing 4 per cent w/v sodium tripolyphosphate (STP) was determined. One part of a solution of the enzyme in tap water is mixed with 3 parts of a solution of STP in distilled water preheated to 50°C, the mixture having a pH of 9.3. The mixture is then placed in a water bath at 50°C for 30 min. After the incubation the residual activity is determined. Approx. 85% of the original activity remained.

Proteolytic activity: A semi-quantitative test for proteinase revealed that the enzyme preparation containing appreciable proteolytic activity at pH 10.

Example 2

Bacillus licheniformis NCIB 8059 was cultivated at 30°C on a rotary shaking table (220 rpm) in 500 ml baffled Erlenmeyer flasks containing 100 ml medium of the following composition:

Potato starch	200 g/l	tap	water
Soybean flour	75 g/l	„	„
$Na_2HPO_4.12H_2O$	9 g/l	„	„
KH_2PO_4	1.5 g/l	„	„
Pluronic	0.1 ml/l	„	„

The word "Pluronic" is a Trade Mark of Wyandotte Chemical Corporation for a polyoxypropylene-polyoxyethylene-condensate.

The medium is liquefied with alpha-amylase and sterilized at 120°C for 90 min.

After 7 days of cultivation the amylase yield was 120 units per ml.

The alpha-amylase activity against soluble starch (Merck amylum solubile) was determined at different pH values using the modified SKB method referred to above.

The results are expressed as per cent of the activity at pH 5:

pH	5	6	7	8	9	10
	100	100	97	95	50	18

The stability in a solution containing 4 per cent w/v sodium tripolyphosphate (STP) was determined. One part of culture fluid (if necessary diluted with tap water) is mixed with 3 parts of a solution of STP in distilled water preheated to 50°C, the mixture having a pH of 9.3. The mixture is then placed in a water bath at 50°C for 30 min. After the incubation the residual activity is determined.

Approx. 60 per cent of the original activity remained.

A semi-quantitative test for proteinase revealed that the culture fluid contained appreciable proteolytic activity at pH 10.

Example 3

Bacillus licheniformis ATCC 6634 was cultivated at 30°C on a rotary shaking table (220 rpm) in 500 ml baffled Erlenmeyer flasks containing 100 ml medium of the following composition:

Potato starch	40 g/l	tap	water
Ground barley	100 g/l	„	„
Soybean flour	30 g/l	„	„
Na_2SO_4	1 g/l	„	„
$CaCO_3$	4 g/l	„	„
Pluronic	0.1 m/l	„	„

The medium is liquefied with alpha-amylase and sterilized at 120°C for 45 min.

After 6 days of cultivation the amylase yield was 30 units per ml.

The alpha-amylase activity was determined at different pH values.

The results are expressed as per cent of the activity at pH 6:

pH	5	6	7	8	9	10
	<35	100	98	98	55	<35

A semi-quantitative test for proteinase revealed that the culture fluid contained appreciable proteolytic activity at pH 10.

Example 4

Bacillus licheniformis ATCC 6598 was cultivated at 30°C on a rotary shaking table (220 rpm) in 500 ml baffled Erlenmeyer flasks containing 100 ml medium of the following composition:

Potato starch	40 g/l	tap	water
Ground barley	100 g/l	„	„
Soybean flour	30 g/l	„	„
Na_2SO_4	1 g/l	„	„
$CaCO_3$	4 g/l	„	„
Pluronic	0.1 ml/l	„	„

The medium is liquefied with alpha-amylase and sterilized at 120°C for 45 min.

After 6 days of cultivation the amylase yield was 450 units per ml.

The alpha-amylase activity was determined at different pH values.

The results are expressed as per cent of the activity at pH 6:

pH	5	6	7	8	9	10
	50	100	100	93	38	13

The stability in a solution containing 4 per cent STP was determined as mentioned in Example 2.

Approx. 62 per cent of the original activity remained.

A semi-quantitative test for proteinase revealed that the culture fluid contained low proteolytic activity at pH 10.

Example 5

Bacillus licheniformis ATCC 11945 was cultivated at 30°C on a rotary shaking table (220 rpm) in 500 ml baffled Erlenmeyer flasks containing 100 ml medium of the following composition:

Potato starch	40 g/l	tap	water
Ground barley	100 g/l	„	„
Soybean flour	30 g/l	„	„
Na_2SO_4	1 g/l	„	„
$CaCO_3$	4 g/l	„	„
Pluronic	0.1 ml/l	„	„

The medium is liquefied with alpha-amylase and sterilized at 120°C for 45 min.

After 6 days of cultivation the amylase yield was 90 units per ml.

The alpha-amylase activity was determined at different pH values.

The results are expressed as per cent of the activity at pH 6:

pH	5	6	7	8	9	10
	40	100	93	78	48	<10

The stability in a solution containing 4 per cent STP was determined as mentioned in Example 2.

Approx. 76 per cent of the original activity remained.

A semi-quantitative test for proteinase revealed that the culture fluid contained appreciable proteolytic activity at pH 10.

Example 6

Bacillus licheniformic ATCC 8480 was cultivated at 30°C on a rotary shaking table (220 rpm) in 500 ml baffled Erlenmeyer flasks containing 100 ml medium of the following composition:

Potato starch	100 g/l	tap	water
Ground barley	50 g/l	„	„
Soybean flour	20 g/l	„	„
Sodium caseinate	10 g/l	„	„
$Na_2HPO_4.12H_2O$	9 g/l	„	„
Pluronic	0.1 ml/l	„	„

The medium is liquefied with alpha-amylase and sterilized at 120°C for 45 min.

After 6 days of cultivation the amylase yield was 140 units per ml.

The alpha-amylase activity was determined at different pH values.

The results are expossed as per cent of the activity at pH 6:

pH	5	6	7	8	9	10
	35	100	100	95	52	16

The stability in a solution containing 4 per cent STP was determined as mentioned in Example 2.

Approx. 70 per cent of the original activity remained.

A semi-quantitative test for proteinase revealed that the culture fluid contained appreciable proteolytic activity at pH 10.

Example 7

Bacillus licheniformis ATCC 9945a was cultivated at 30°C on a rotary shaking table (220 rpm) in 500 ml baffled Erlenmeyer flasks

containing 100 ml medium of the following composition:

Potato starch	100 g/l	tap	water
Ground barley	50 g/l	„	„
S ybean flour	20 g/l	„	„
Sodium caseinate	10 g/l	„	„
$Na_2HPO_4.12H_2O$	9 g/l	„	„
Pluronic	0.1 ml/l	„	„

The medium is liquefied with alpha-amylase and sterilized at 120°C for 45 min.

After 7 days of cultivation the amylase yield was 40 units per ml.

The alpha-amylase activity was determined at different pH values.

The results are expressed as per cent of the activity at pH 6:

pH	5	6	7	8	9	10
	90	100	97	96	77	50

A semi-quantitative test for proteinase revealed that the culture fluid contained appreciable proteolytic activity at pH 10.

Example 8

The advantage of a *B. licheniformis* amylase over a *B. subtilis* amylase for dishwashing purposes is shown in the following example.

L is a *B. licheniformis* amylase
S is a commercial amylase produced from *B. subtilis.*

Tap water (250 ml), NaCl (1.0 g), milk (70 ml) and mashed potato powder (30 g), are mixed together and then heated for 1 minute at 100°C, whereafter the mass is stirred until homogenous. Then defatted watch glasses are each evenly coated with 0.5 g of the mixture and dried at 50°C for 30 minutes.

A detergent having the following composition by weight is prepared:

Sodium tripolyphosphate	25%
Sodium meta silicate	20%
Tri sodium phosphate	10%
Sodium bicarbonate	42%
Tenside (ethoxylated nonylphenol)	3%

The detergent is used in 0.2% w/v concentration in tap water.

1 litre samples of the detergent solution are pre-heated to 60°C, enzyme is added in appropriate amounts and the mixture transferred to beakers equipped with agitation and placed in water bath thermostated at 60°C.

The watch glasses are immersed in the enzyme-detergent solution and heated for 20 minutes at 60°C with agitation (70 rpm).

After washing the glasses are taken up and after rinsing and drying examined visually. The results are recorded using a graduated scale in which "0" signifies no change, and "5" complete cleaning of the watch glasses.

The following results were obtained:

Enzyme concentration in units per litre:	0	500	1500	2500
Enzyme L	1	4	5	5
Enzyme S	1	2	3	4

Liquification with alpha-amylase utilises the technique basically well known in the art: since a substrate contains starch, on heating gelatinization will take place and the viscosity will increase to a very high value. This means that stirring and thereby also aeration will become extremely difficult or even impossible. Thus, the viscosity must be lowered and this is done by adding amylase, e.g. amylase prepared from *B.subtilis* or *B.licheniformis*, when the substrate temperature is between 65 and 95°C. Such an amount of amylase is added that the substrate viscosity is lowered to a level where stirring can easily be instituted.

WHAT WE CLAIM IS:—

1. A process for the production of an enzyme product containing an alpha-amylase, which process comprises cultivating a microorganism of the species *Bacillus licheniformis* in a nutrient medium for the microorganism to produce an enzyme product.

2. A process according to Claim 1, wherein the enzyme product is isolated from the fermentation broth.

3. A process according to Claim 1 or 2, wherein the strain *Bacillus licheniformis* NCIB 8061 is used.

4. A process according to Claim 1 or 2, wherein a strain of *Bacillus lichiformis* selected from NCIB 8059, ATCC 6634, ATCC 6598, ATCC 11945, ATCC 8480 and ATCC 9945a is used.

5. A process for the production of an enzyme product, substantially as described in foregoing Example 1.

6. A process for the production of an enzyme product substantially as described in foregoing Example 2.

7. A process for the production of an enzyme product substantially as described in foregoing Example 3.

8. A process for the production of an

enzyme product substantially as described in foregoing Example 4.

9. A process for the production of an enzyme product substantially as described in foregoing Example 5.

10. A process for the production of an enzyme product substantially as described in foregoing Example 6.

11. A process for the production of an enzyme product substantially as described in foregoing Example 7.

12. An enzyme product, whenever prepared by the process of any one of Claims 1 to 11.

13. The use of alpha-amylase according to Claim 12 in a cleaning composition.

14. The use of alpha-amylase according to Claim 12 in a dishwashing composition.

FORRESTER, KETLEY & CO.,
Chartered Patent Agents,
Jessel Chambers, 88/90 Chancery Lane,
London, WC2A 1HB.
and
Rutland House, Edmund Street,
Birmingham, B3 2LD.
Agents for the Applicants.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1972.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.